

Identification and characterization of novel bioactive peptide from red seaweed (*Pyropia vietnamensis*) proteins

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Abstract

Pyropia vietnamensis is one of the most abundant seaweeds in the Indo-Pacific region. This study aimed to perform an *in silico* evaluation of *P. vietnamensis* proteins as potential precursors of bioactive peptides and to determine the novel peptide in terms of its half maximal inhibitory concentration (IC₅₀) and the stability under a controlled laboratory environment (*in vitro*) toward the dominant biological activity. The proteomic profiles of *P. vietnamensis* proteins were determined using LC-MS/MS analysis. Next, five proteins were chosen and employed for *in silico* analysis using the BIOPEP-UWM database. The *in vitro* characterizations of novel peptides were carried out using a dipeptidyl peptidase-IV (DPP-IV) inhibitor screening assay kit. *In silico* analysis revealed that DPP-IV and angiotensin-converting enzyme (ACE) inhibitors were the most potential bioactive peptides in *P. vietnamensis* proteins. Calpain 2, papain, pepsin (pH > 2), and stem bromelain were predicted as the enzymes with the most potential to produce DPP-IV and ACE inhibitors. The novel peptides predicted were CFA, ACE, RFPS, DEWG, NYCL, CVPR, and DACE. The synthesized CVPR with an IC₅₀ of 0.66 mg/ml exhibited stability at pH 3–7, 30–50°C, and resisted gastrointestinal digestion. This study revealed *P. vietnamensis* proteins could offer health benefits due to their therapeutic potential with sustainable industrial applications on functional foods, nutraceuticals, and pharmaceuticals.

Keywords: ACE inhibitory peptide; antidiabetes; antihypertensive; DPP-IV inhibitory peptide; *in silico*

Introduction

Seaweeds are classified into red, green, and brown based on their pigmentations, nutrients, and chemical compositions (Sultana *et al.*, 2023). They have been consumed as food mainly in China, Japan, and the Republic of

Korea. According to El-Beltagi *et al.* (2022), seaweeds are increasingly utilized in the medical field due to their diverse structural features and novel biological activities including antihypertensive, antidiabetic, antimicrobial, anticancer, and antioxidant properties. *Pyropia*, formerly known as *Porphyra*, accounted for more than 95%

of cultivated seaweed globally in 2019, and Malaysia is one of the top 10 producers. Several *Pyropia* species are used to prepare commercial food called “nori,” which is widely consumed by the Japanese. Bioactive peptides are protein fragments composed of 2–20 amino acids that exhibit health-promoting properties in the human body (Agirbasli and Cavas, 2017). The protein content of red seaweeds is the highest (up to 47%) compared to green (9–26%) and brown seaweeds (3–15%) (Peñalver *et al.*, 2020). Hence, red seaweeds could be a good precursor for the generation of bioactive peptides. The identification of bioactive peptides from food proteins can be determined either via *in vitro* or *in silico* approach. This study utilized an *in silico* approach using the BIOPEP-UWM database that contained information on bioactive peptides including their protein sequences and biological properties. The *in silico* method is important in the prediction of potential biological activity and enzyme substrate to release specific bioactive peptides.

In the advanced research of bioactive peptides, *in silico* methods enable efficient screening, design, and characterization of peptides. The integration of *in silico* with experimental methods may accelerate the development of bioactive peptides for diverse applications, such as functional foods, nutraceuticals, and pharmaceuticals. The key applications of the *in silico* method include peptide discovery and screening, peptide design and optimization, molecular docking and dynamic simulation, and structure-activity relationship (SAR) analysis. Potential bioactive peptides released from the proteins can be commercialized as functional foods, nutraceuticals, and pharmaceuticals targeted for certain bioactivity. For example, Nori S peptide (Shirako Co., Ltd., Tokyo, Japan) and Wakame peptide jelly (Riken Vitamin Co., Ltd., Tokyo, Japan) are functional products from seaweed produced in Japan with FOSHU-approved antihypertensive claims (Hayes and Tiwari, 2015).

Generally, this study was conducted to fill gaps in current knowledge that limit the full understanding and application of seaweed-derived peptides in managing metabolic disorders. For example, there is limited diversity in peptide identification and isolation where only a small fraction of the vast diversity of seaweed peptides has been explored especially DPP-IV inhibition. Also, the variability in levels of bioactivity between seaweed species is poorly understood, for instance, *Palmaria palmata* possessed potent DPP-IV inhibitory activity (Harnedy *et al.*, 2015). Hence, it is anticipated to observe the factors influencing this variability including environmental conditions and extraction method of *Pyropia vietnamensis*. Additionally, this study paves the way for a deeper understanding of peptide stability under physiological conditions such as pH, temperature, and gastrointestinal digestion which affects their potential bioavailability and

efficacy. Importantly, the advanced screening techniques used in this study are valuable in research directions to address gaps. Recently, the antioxidant and antimicrobial properties of *P. vietnamensis* have been reported by Bhatia *et al.* (2021) *in vitro*. To the best of our knowledge, no study has been reported on proteomic profiles of *P. vietnamensis* proteins and the identification of bioactive peptides from it using *in silico* approach. Therefore, this study aims to determine the proteomic profiles of *P. vietnamensis* proteins, to identify its most promising bioactive peptides using an *in silico* approach and to perform *in vitro* characterization of a synthesized novel peptide.

Materials and Methods

Materials and chemical reagents

Around 1 kg of *P. vietnamensis* was collected from the upper intertidal zone at Tok Jembal Beach, Kuala Nerus, Terengganu, Malaysia during the monsoon season (low tide). Liquid nitrogen was purchased from MCY Marine (Kuala Terengganu, Terengganu, Malaysia). Phenol, dithiothreitol (DTT), urea, pancreatin (EC232.468.9), formic acid, bovine trypsin (proteomic grade), and dipeptidyl peptidase IV (DPP-IV) Inhibitor Screening Kit were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Pepsin (EC3.4.23.1), ammonium acetate, and methanol were purchased from R&M Chemicals (Essex, UK). The Coomassie (Bradford) Protein Assay Kit was purchased from Thermo Fisher (Rockford, Illinois, USA). All other chemicals used were of analytical grades.

Preparation of sample

A schematic diagram of the methodology has been illustrated in Figure 1. Firstly, the mature *P. vietnamensis* was carefully selected and collected by removing the dried and premature ones. The sample was thoroughly rinsed with clean seawater to eliminate debris, sand, epiphytes, and other extraneous substances and was delivered to the laboratory in an ice box. In the laboratory, *P. vietnamensis* were rinsed with distilled water and dried with tissue paper to remove excess water. The cleaned seaweed samples were deep-frozen in liquid nitrogen for rapid freezing and crushed directly in liquid nitrogen into a fine powder using a prechilled mortar and pestle, before freezing at -80°C . Next, acetone-dried powder (AcDP) of seaweed was prepared according to Awang *et al.* (2010) with some modifications. Seaweed powder (± 5 g/tube) was washed with ice-cold acetone (10 mL/tube) using vortex for 5 min and was then centrifuged at $10,000 \times g$ for 5 min at 4°C before the supernatant was discarded. This washing step was carried out until the color of ice-cold acetone was clear to ensure that the contaminants, for example, lipids, polysaccharides, phenolic compounds, and pigments,

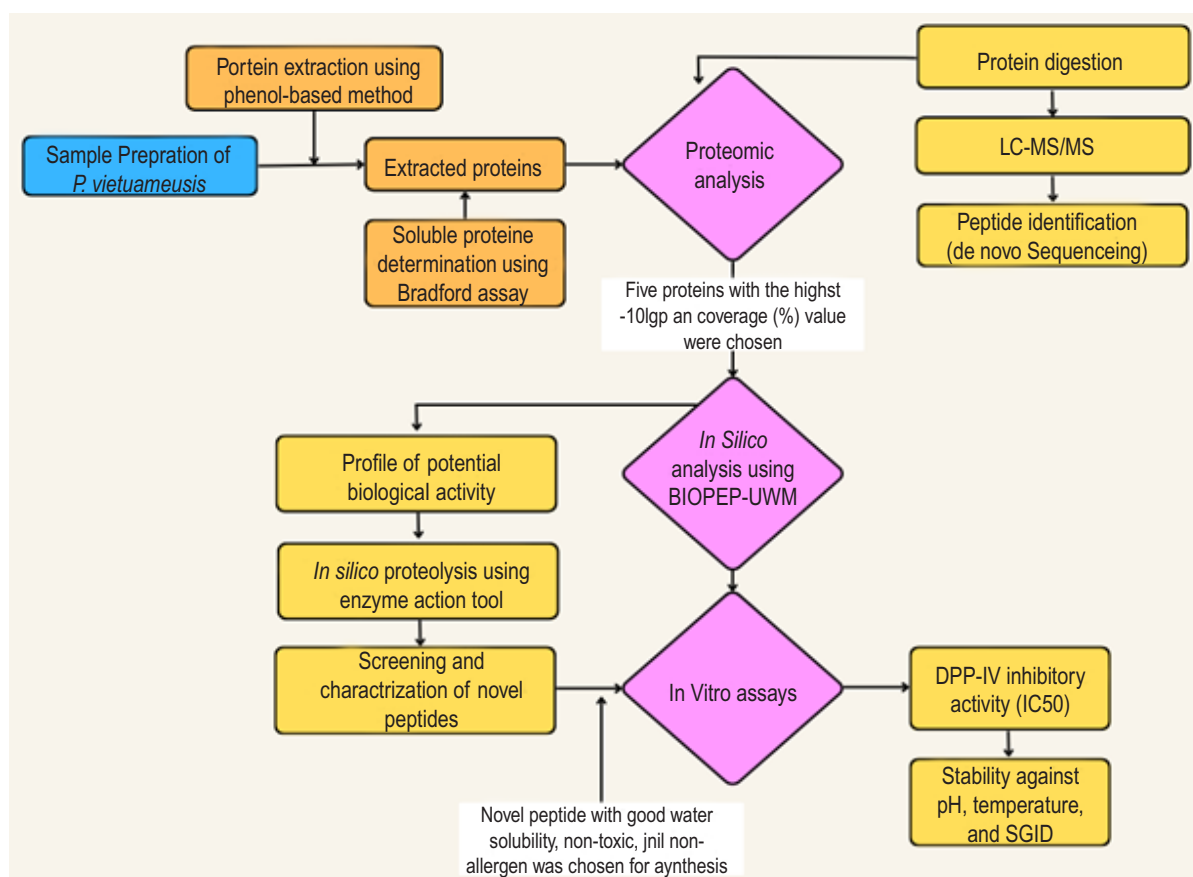


Figure 1. A schematic diagram of the methodology.

were completely removed from the sample, to facilitate protein extraction. In this study, the washing step was repeated nine times to get a clear color of ice-cold acetone. Next, the remaining ice-cold acetone in the resulting pellet was allowed to evaporate at room temperature. AcDP was stored at -80°C in a sealed container for further analysis.

Protein extraction

Protein extraction was performed using the phenol extraction methanol–ammonium acetate precipitation (PHE) method as described by Carpentier *et al.* (2005) and Awang *et al.* (2010) with some modifications. AcDP (1.0 g) was resuspended in 10 mL of extraction buffer (50 mM Tris-HCl pH 8.5, 0.5 M EDTA, 100 mM KCl, 1% v/v DTT, 30% w/v sucrose) and was mixed using vortex mixer for 30 s. Next, ice-cold Tris-buffered (pH 8.0) phenol (10 mL) was added, and the mixture was mixed using a vortex mixer for 15 min, followed by centrifugation ($10,000 \times g$ for 3 min) at 4°C . The phenolic phase was collected and reextracted with an equal volume of extraction buffer. The phenolic phase after centrifugation was allowed to precipitate overnight with five times the volume of cooled 100 mM ammonium acetate in methanol at -20°C . Then, the supernatant

was discarded after centrifugation ($10,000 \times g$, 30 min at 4°C). The resulting pellet was washed twice with ice-cold acetone containing 0.2% v/v DTT and between the two rinsing steps, the sample mixture was incubated for 1 h at -20°C . During each wash, the pellet was vortexed for 10 min at 4°C to remove pigments and other interfering compounds properly. The pellets were air-dried at room temperature ($26^{\circ}\text{C} \pm 1$). The pellets were resuspended in 300 μL resolubilization buffer containing 7 M urea, 2 M thiourea, 4% v/v CHAPS, and 1% v/v DTT. The sample mixture was gently vortexed every 15 min for 1 h at room temperature. The supernatant was collected after centrifugation at $10,000 \times g$ for 30 min at 20°C and was stored at -80°C for further analysis. Total soluble protein in the supernatant was determined using the Bradford assay (Bradford, 1976), on 96-well microtiter plates by referring to the Thermo Scientific Coomassie (Bradford) Protein Assay Kit Instructions. It was found that the soluble protein content of *P. vietnamensis* was 14.90 mg/mL ($n = 4$).

LC-MS/MS analysis and database searching

Before LC-MS/MS analysis, the extracted protein sample was digested using the trypsin in-solution digestion

method (Kinter and Sherman, 2005). The sample was then mixed with 100 µL of 0.1% formic acid in deionized water. The solution was filtered using a 0.45 µm regenerated cellulose (RC) membrane syringe filter (Sartorius AG, Goettingen, Germany). The LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, CA, USA) coupled with Easy-nLC II nano-liquid chromatography system was used to perform the analysis. The eluent was sprayed into the mass spectrometer at 2.1 kV (source voltage) at 220°C. Full scan mass analysis was performed from 300–2000 m/z at a resolving power of 60,000 (at m/z 400, FWHM; 1-s acquisition) with data-dependent MS/MS analyses (ITMS) triggered by the eight most abundant ions from the parent mass list of predicted peptides with rejection of singly and unassigned charge state. Collision-induced dissociation (CID) was applied as the fragmentation technique. Collision energy was set at 35. The sample was analyzed in duplicate readings.

PEAKS Studio software (PEAKS, version 7.5, Bioinformatics Solution, Waterloo Canada) was used to perform de novo sequencing and database searching. The peptide sequences were matched with proteins from the UniProtKB (Seaweed and Algae) database. Parent mass and precursor mass tolerance were set at 0.1 Da. The false discovery rate (FDR) of protein and peptide detection was set at <0.1% to increase confidence. In addition, the significant score of –10I gP for proteins greater than 20 was used for protein acceptance. At the same time, the minimum number of the unique peptide was set as to 1.

Protein sequences of red seaweed (*P. vietnamensis*)

Five proteins from LC-MS/MS results were selected based on the highest confidence level (–10I gP) values (240.79–162.24) and the protein sequences were confirmed with the Universal Protein Knowledgebase (UniProtKB, <https://www.uniprot.org/>) library database.

In silico analysis using BIOPEP-UWM database

Evaluation of *P. vietnamensis* as a potential precursor for bioactive peptides

The probability of five selected *P. vietnamensis* proteins to liberate bioactive peptides was analyzed using the BIOPEP-UWM database. Accessed on January 14, 2023, this database contains 4623 acknowledged bioactive peptides with 62 biological activities. The number of potential bioactive peptides of each protein was computed using “profiles of potential biological activity” in the database and the predicted biological activity was analyzed altogether. The data obtained were tabulated in

Microsoft Excel 2021 and the predicted fragments of specific biological activity to be released from the selected proteins were counted manually. The biological activity that gave the highest number of fragments was chosen to be reported.

The frequency of occurrence of fragments with given activity (A) of *P. vietnamensis* proteins was calculated by the database using the following formula, where a is the number of fragments of given activity in a protein sequence, and N is the number of amino acid (AA) residues in the protein chain. The total frequencies of occurrences of fragments (ΣA) for each protein sequence were also calculated by the database.

$$A = \frac{a}{N}$$

The BIOPEP-UWM database also shows the potential biological activity of the protein (B) of proteins and was calculated by using the following formula, where a is the number of fragments of given activity in a protein sequence, and N is the number of amino acid (AA) residues in the protein chain.

$$B = \frac{\sum_{i=1}^k \frac{a_i}{EC_{50i}}}{N}$$

In silico proteolysis

Pyropia vietnamensis proteins were subjected to *in silico* proteolysis using BIOPEP's enzyme-action tool. Figure 2 shows the flowchart of *in silico* proteolysis conducted in this study. Each protein sequence was hydrolyzed individually by 33 different enzymes contained in the database. The potential enzymes to release bioactive peptides with diverse biological activities from the proteins were selected to report including pancreatic elastase, papain, ficin, leukocyte elastase, stem bromelain, calpain 2, and pepsin (pH > 2). Enzyme combinations were performed among those seven selected enzymes. The efficiency of the released fragments was measured based on the frequency of occurrence of fragments by selected enzymes (A_E) using the following formula, where d is the number of fragments with a given activity in the protein sequence released by enzymes, and N is the number of amino acid residues in protein chains. The sum of frequencies of occurrences (ΣA_E) of all *P. vietnamensis* proteins for DPP-IV inhibitory and ACE inhibitory activities using a single and combination of two or three enzymes was summarized in Figure 3.

$$A_E = \frac{d}{N}$$

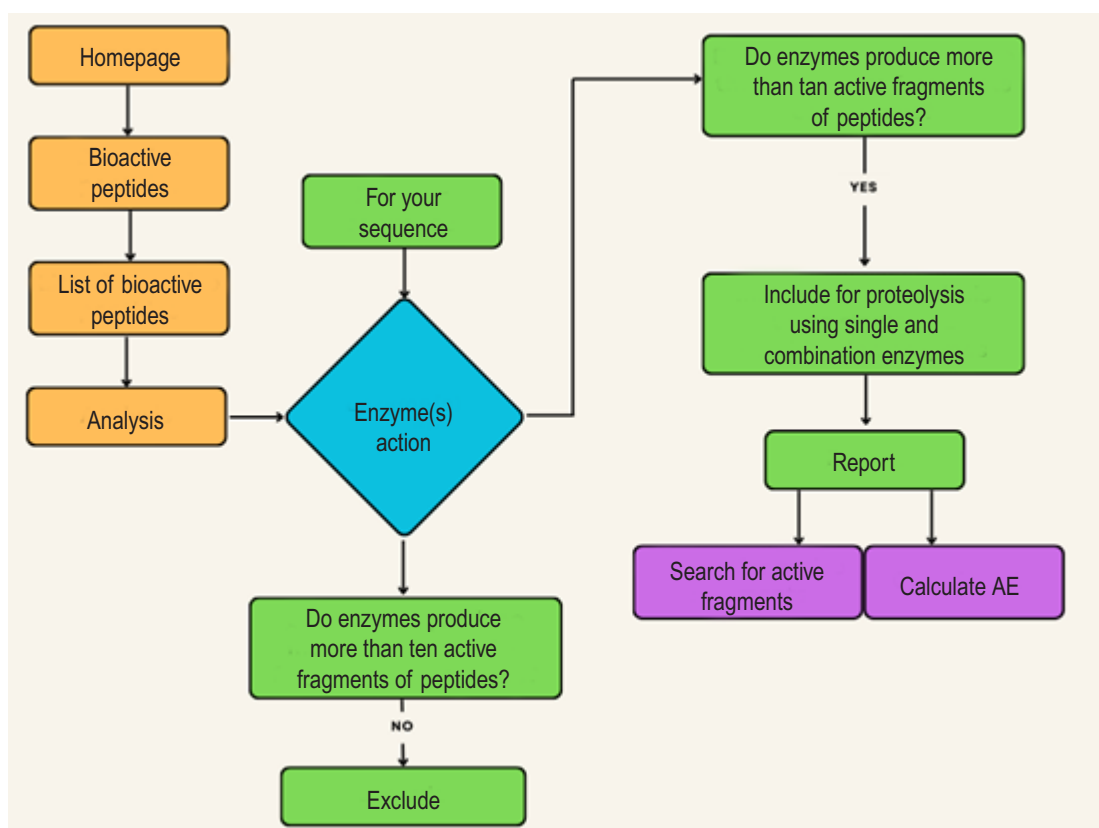


Figure 2. Scheme of *in silico* proteolysis using enzyme-action tool of BIOPEP-UWM database.

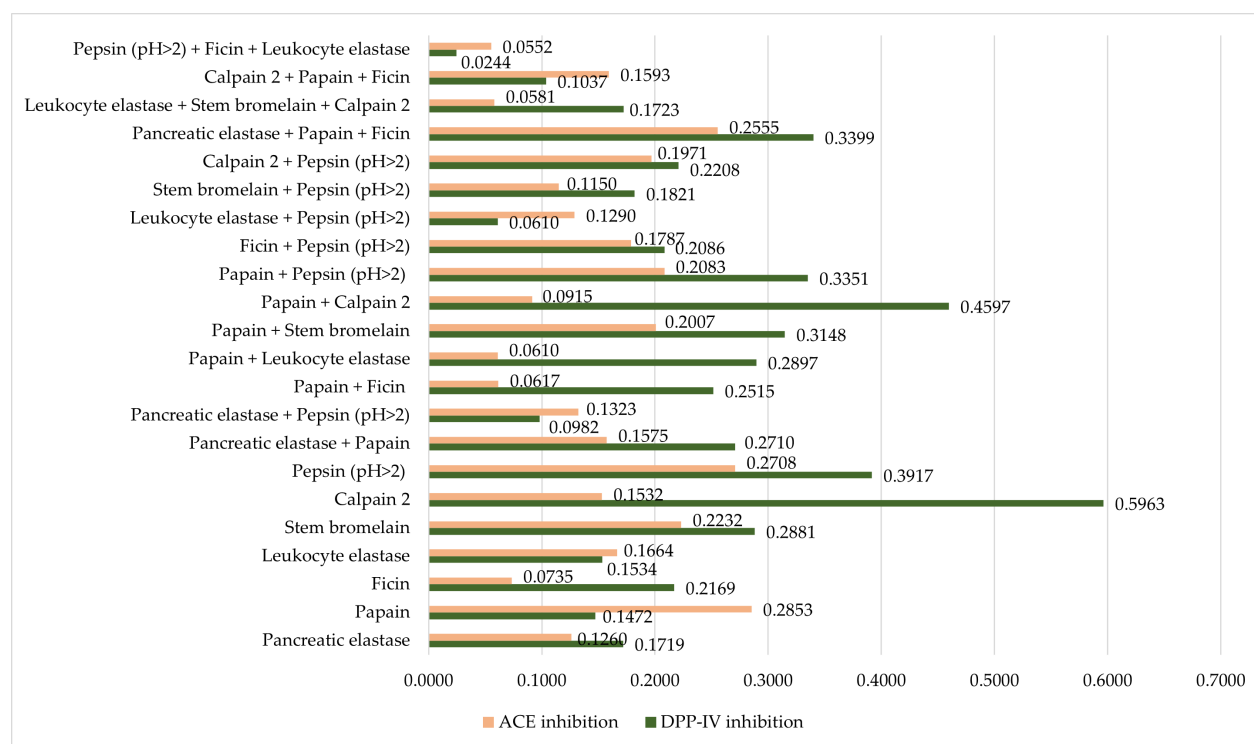


Figure 3. The sum of frequencies of occurrences (ΣA_E) of DPP-IV inhibitor and ACE inhibitor using single and combination enzymes.

In silico screening and characterization of novel peptides

The peptide fragments predicted to be released from *P. vietnamensis* proteins with known biological activity were counted manually. BIOPEP-UWM shows the fragments with the activities available in the database. In this study, sequences of three and four amino acid residues were screened and considered for potential biological activity. The bioinformatics tool used to measure the bioactivity score for peptide fragments was PeptideRanker which is accessible at <http://distilldeep.ucd.ie/PeptideRanker>. It is based on the prediction and ranking of the likelihood of a peptide exerting bioactive properties based on an N-to-1 neural network algorithm. According to Coscueta *et al.* (2022), a peptide that scores above the PeptideRanker threshold (0.5) is identified as bioactive. Thus, novel peptides with a bioactivity score >0.5 were considered for analysis as potentially bioactive. *In silico* characterizations in terms of water solubility, resistance to digestion, toxicity, allergenicity, and IC₅₀ were conducted to determine the potential characteristics of novel peptides. The solubility of novel peptides in water was predicted using PepCalc at <http://pepcalc.com>. Gastrointestinal digestion was predicted using PeptideCutter, available at http://webexpasy.org/peptide_cutter. Three different enzymes; chymotrypsin-low specificity, chymotrypsin-high specificity, pepsin (pH 1.3), pepsin (pH > 2), and trypsin were used to evaluate the resistance of the novel peptides against digestion. Next, ToxinPred and AllerTop were utilized to determine the toxicity and allergenicity of the novel peptides, which are freely accessible at <http://www.imtech.res.in/raghava/toxinpred/> and <https://www.ddg-pharmfac.net/AllerTOP>, respectively.

In vitro analyses

The selected novel tetrapeptide (CVPR) was synthesized to further analyze *in vitro*. The synthesis was not performed by the authors of the article but outsourced to the commercial company Apical Scientific Sdn. Bhd. (Malaysia). This synthetic peptide with purity of 99.1% was used to determine its DPP-IV inhibitory activity and stability.

DPP-IV inhibitory activity

The activity of synthetic DPP-IV inhibitory peptide was determined using a DPP-IV Inhibitor Screening Kit (Sigma-Aldrich, USA) in 96-well plates. All assays were performed in triplicate (n = 3) and Sitagliptin was used as a positive control. The DPP-IV inhibitory activity was determined by plotting % inhibition as a function of the concentration of the test compound. The values were expressed as the mean of half maximal inhibitory concentration (IC₅₀) that inhibits DPP-IV activity by 50% ± standard deviation. The DPP-IV inhibitory activity was

calculated as follows, where FLU1 is the fluorescence at T1; FLU2 is the fluorescence at T2; SlopeSM is the slope for the inhibitor sample and SlopeEC is the slope for enzyme control.

$$\text{Slope} = \frac{(\text{FLU2} - \text{FLU1})}{(T2 - T1)}$$

$$\text{Relative inhibition} = \frac{(\text{SlopeEC} - \text{SlopeSM})}{\text{SlopeEC}} \times 100\%$$

Stability of a synthetic DPP-IV inhibitory peptide

The stability of a synthetic DPP-IV inhibitory peptide against pH, temperature, and simulated gastrointestinal digestion was determined according to the method by Kong *et al.* (2021) and Walsh *et al.* (2004) with minor modifications. For pH stability, the peptide was diluted with deionized water to a working concentration of 2 mg/mL. Next, 10 mL of peptide solution was adjusted to different pH values (3.0, 5.0, 7.0, 9.0, or 11.0, respectively) and incubated in a water bath at 25°C for 30 min. The pH of the treated samples was immediately adjusted to pH 7. The sample without pH treatment was used as a control.

For temperature stability, the peptide was diluted with deionized water and made up to 2 mg/mL working concentration. Next, 10 mL of peptide solution was heated at different temperatures (30°C, 50°C, 70°C, or 90°C, respectively) in a water bath (Memmert GmbH, Germany) for 30 min. All samples were immediately cooled in iced water. The sample without heat treatment (25°C) was used as a control.

For stability against simulated gastrointestinal digestion (SGID), the peptide was diluted to 10 mg/mL working concentration and reduced to pH 2.0 using 1 N HCl. Then, pepsin was added to the peptide solution with a ratio of an enzyme to the substrate of 1:40 (w/w) and the reaction mixture was incubated in a water bath shaker for 90 min at 37°C. The pH of the reaction mixture was then adjusted to pH 7.5 by the addition of 1 M sodium hydroxide (NaOH) and was further incubated with pancreatin at an enzyme-to-substrate ratio of 1:25 (w/w) in a water bath shaker at 37°C. After 150 min, the reaction mixture was exposed to 80°C for 20 min to stop the digestion. The DPP-IV inhibitory activity was measured and expressed as the activity (%) relative to that without any treatment (control, 100%) for all of the assays above.

Statistical analysis

All results were presented as mean ± standard deviation. Data were subjected to One-Way analysis of variance (ANOVA) by using SPSS Version 20.0 (SPSS Inc.,

Chicago). Significant difference between treatments were analyzed further using least significant difference (LSD) at 0.05 significant level.

Results

Potential bioactive peptides from *P. vietnamensis* proteins

Protein sequences in FASTA format and common features for selected proteins from *P. vietnamensis* are listed in Table 1. The amino acid residues of these proteins range from 161–172 with a molecular mass of 17,464 – 18,201 Da. Protein C-phycoerythrin beta chain had the highest amino acid residues (172) and the highest molecular mass (18,201 Da). Table 2 shows the total number of potential bioactive peptides from *P. vietnamensis* proteins.

Table 3 shows the frequency of occurrence (A) and potential biological activity (B) values of bioactive peptides from *P. vietnamensis* proteins. DPP-IV inhibitor exhibited a higher A value for all selected proteins, followed by ACE inhibitor. Therefore, this study focused on the DPP-IV inhibitor and ACE inhibitor as the main biological activity to be released from *P. vietnamensis* proteins. The total frequency of occurrences of bioactive fragments (ΣA) among five proteins ranged from 1.3081 to 1.6646. Based on the data, the allophycoerythrin beta chain showed the highest value followed by the phycoerythrin alpha subunit, allophycoerythrin alpha chain, r-phycoerythrin alpha chain, and c-phycoerythrin beta chain. The highest A value of the DPP-IV inhibitor in the allophycoerythrin beta chain was observed to contribute to the majority of ΣA value. The database also presented the potential biological activity of protein (B). In contrast,

the B value of the ACE inhibitor was higher than that of the DPP-IV inhibitor.

In silico proteolysis of *P. vietnamensis* proteins

Enzymatic hydrolysis is the most common method to release peptides from parent proteins, enabling them to exhibit biological functions. This method requires the use of one or more enzymes to react with sample proteins to release bioactive peptides. *In silico* proteolysis was conducted to predict the potential proteolytic enzymes capable of producing peptides with specific biological activity. In this study, selected proteins were subjected individually to 33 different enzymes contained in the BIOPEP-UWM database using an enzyme-action tool (not shown). Among the 33 different enzymes, seven enzymes namely pancreatic elastase, papain, ficin, leukocyte elastase, stem bromelain, calpain 2, and pepsin (pH > 2) were identified as potential candidates for releasing bioactive peptides from the proteins, mainly DPP-IV inhibitors. Hence, these enzymes were selected for further reporting. Enzymatic action generated multiple peptides from each protein. Table 4 shows the number of potential DPP-IV inhibitory and ACE inhibitory fragments to be released from *P. vietnamensis* proteins by specific enzymes. These peptides, which have been reported and exist in the database, were predominantly dipeptides. In addition, tripeptides (GPV) and tetrapeptides (ASL, AEL, SVYT) were also found. Among the selected enzymes, calpain 2 and pepsin (pH > 2) were predicted to release the most peptide fragments for both DPP-IV inhibitors and ACE inhibitors.

The efficiency of selected enzymes in releasing bioactive peptides was measured based on the frequency of release

Table 1. Accession numbers for protein database and sequences of protein from seaweed.

Protein (Accession number)	Sequence	Amino acid length	Molecular mass (Da)
R-phycoerythrin alpha chain (P51368)	MKSVITTTISAADAAGRFPSSSDLESVQGNQIRAAARLEAAEKLASNHEAV VKEAGDACFAKYSYLKNPGEAGDSQEKVNKCYRDVDHYMRLVNYCLVGGTGP VDEWGIAGAREVYRTLNLPTSAYVASFAFARDRLCVPRDMSAQAGVEYAGNLDYIINSLC	164	17,698
Phycocyanin alpha subunit (A0A141SF63)	MKTPITEAIASADSQGRFLSNGELQAINGRYQRAAASLGAARSLTNNARLITGAAQS VYTKFPYVTQMPGPTYASSAIGKAKCARDIGYYLRMVTYCLVVGATGPMDEYLVAG LEEINRSFELSPSWYVEALQYIKGSHGLSGQIGNEANVYLDYAINTLS	162	17,464
Allophycoerythrin alpha chain (P59856)	MSIVTSKIVNADAEARYLSPGELDRIKSFVLVSGQRRRLRIQILTDNRERIVKQGGQQ LFQKRDPVSPGGNAYGEEMTATCLRDLDYYLRLVTYGVAGDVPTPIEEIGLVGVKE MYNSLGTPISGVAEGVKCMKSVACSLLAGEDSAEAGFYFDYTLGAMQ	161	17,509
C-phycoerythrin beta chain (Q1XDB0)	MLDAFAKVVAAQADARGEFLSNTQLDALSSMVAEGNKRDLVDVNNKINSNASAIVTNSA RALFAEQPQLIQPGGNAYTSRRMAACLRDMEIVLRVYSYAMIAGDSSVLDDRLNGLRE TYQALGTGSSSVSAVQKMKEASVALANDLTGITQGDSCALIAELGSYFDRAAVSVV	172	18,201
Allophycoerythrin beta chain (P59857)	MQDAITSVINAADVQGKYLDSSVEKLGRGYFQTGELRVRAAATAANAATIICESVAKS LLYSDITRPGGNMYTTRRYAACIRDLDDYYLRYATYGLAGDPSILEERVNLGLK ETYNLSLGPVIGATIQAILAMKEVTISLVGPDAGKEMGLYFDYICSGLS	161	17,484

Table 2. Total number of potential bioactive peptides from *P. vietnamensis* proteins.

Proteins	Number of activities	Number of potential bioactive peptides					
		DPP-IV inhibitor	ACE inhibitor	DPP-III inhibitor	Anti-oxidative	Alpha-glucosidase inhibitor	Other activities
R-phycoerythrin alpha chain	15	103	83	15	7	6	26
Phycocyanin alpha subunit	19	99	86	12	18	5	41
Allophycocyanin alpha chain	13	97	77	16	9	3	34
C-phycocyanin beta chain	14	105	71	14	6	2	24
Allophycocyanin beta chain	16	105	84	18	15	2	41
Total number of potential bioactive peptides		509	401	75	55	18	166

ACE, angiotensin-converting enzyme; DPP-III, dipeptidyl peptidase-III; DPP-IV, dipeptidyl peptidase-IV

Table 3. The frequency of occurrence (A) and the potential biological activity (B) of bioactive peptides from *P. vietnamensis* proteins.

Protein	ΣA	The frequency of occurrence of bioactive peptides (A) (The potential biological activity of bioactive peptides [B])				
		DPP-IV inhibitor	ACE inhibitor	DPP-III inhibitor	Antioxidative	Alpha-glucosidase inhibitor
R-phycoerythrin alpha chain	1.4635	0.6280 (0.0002)	0.5061 (0.0214)	0.0915	0.0427	0.0366
Phycocyanin alpha subunit	1.6175	0.6111 (0.0001)	0.5309 (0.0625)	0.0741	0.1111	0.0370
Allophycocyanin alpha chain	1.4845	0.6025 (0.0002)	0.4783 (0.0160)	0.0994	0.0559	0.0373
C-phycocyanin beta chain	1.3081	0.6105 (0.0004)	0.4128 (0.0064)	0.0814	0.0349	0.0291
Allophycocyanin beta chain	1.6646	0.6522 (0.0002)	0.5217 (0.0108)	0.1118	0.0932	0.0311

*The value of the potential biological activity of the protein (B) for specific activities was rounded off to the fourth decimal place. ΣA , sum of the frequency of occurrence of bioactive peptides; ACE, angiotensin-converting enzyme; DPP-III, dipeptidyl peptidase-III; DPP-IV, dipeptidyl peptidase-IV

fragments with a given activity by selected enzymes (AE) and the relative frequency of release fragments with a given activity by selected enzymes (W). Figure 3 illustrates the ΣAE values of DPP-IV inhibitor and ACE inhibitor after undergoing *in silico* proteolysis using a single and combination of enzymes. Among the single enzyme treatments, calpain 2 was predicted to be the most effective in releasing DPP-IV inhibitory peptides with higher A values, while papain seems to be promising in producing ACE inhibitory peptides. Moreover, pepsin (pH > 2) and stem bromelain were also observed as potential enzymes capable of releasing peptides with both DPP-IV and ACE inhibitory activity. Enzyme-action tool allows the proteolysis to be simulated using a combination of enzymes up to three enzymes. As a result, papain + calpain 2, papain + pepsin (pH > 2), and pancreatic elastase + papain + ficin were the potential combinations predicted to produce DPP-IV inhibitory peptides with higher A values compared to the other enzyme combinations. In contrast, combinations of papain + stem bromelain, papain + pepsin (pH > 2), and pancreatic

elastase + papain + ficin had the potential to release ACE inhibitory peptides.

In silico screening and characterization of novel peptides

In this study, unidentified tripeptide and tetrapeptide were screened as novel peptides. The novel peptides could be produced during the digestion of the parent protein (Akbarian *et al.*, 2022) and could possess potent biological activities, allergenic properties, or toxic properties once absorbed into the body cell. Nine tripeptides (QRA, CFA, RDV, DHY, ESV, ACE, AYV, DMS, DYI) and 21 tetrapeptides (RFPS, NHEA, KNPG, QEKV, NKCY, DEWG, RDRL, EVYR, CVPR, NIQR, DACE, DVDH, AQAG, IINS, QGNI, NYCL, SVIT, SNHE, AVVK, EVYR, VPRD) were unreported peptides to be released by the activity of specific enzymes. Narrowing down, seven novel peptides were selected for further investigation as they are potentially bioactive based on the bioactivity score (>0.5) calculated using the PeptideRanker tool, with

Table 4. The number of potential DPP-IV inhibitors and ACE inhibitors to be released by selected enzymes using BIOPEP's enzyme-action tool.

Enzyme	DPP-IV inhibitor	ACE inhibitor
Pancreatic elastase	70	47
Papain	78	52
Ficin	55	38
Leukocyte elastase	62	36
Stem bromelain	77	58
Calpain 2	98	63
Pepsin (pH >2)	109	61

the most likely being CFA, ACF, and DACF (Table 5). This online tool calculates bioactivity scores based on the neural network model. This model is trained on a dataset containing peptides with experimentally verified bioactivities and inactive peptides. It enables the tool to learn patterns and features that are associated with bioactivity. The model then evaluates the peptide sequence and assigns a bioactivity probability score between 0 and 1, with 0 indicating a low likelihood of bioactivity, while 1 indicates a high likelihood of bioactivity. Commonly, peptides with scores above 0.5 are considered to have significant bioactivity potential (Coscueta *et al.*, 2022).

Based on the results, the water solubility of CFA, ACF, and NYCL was poor, which may be due to their hydrophobic residues. Interestingly, all peptides exhibited nontoxic and nonallergenic properties, except for RFPS and NYCL. Nevertheless, all of them are predicted to degrade following enzymatic digestion. Furthermore, this study

primarily focused on DPP-IV inhibitory activity, and the potential novel peptide was chosen for *in vitro* analysis. Among the novel peptides, CVPR was chosen to be further characterized *in vitro* due to its good water solubility, which would facilitate the experiment and indicate its potential availability in human blood after crossing cell membranes. This criterion is important for the peptide to exhibit biological effects in the human body. Although CFA and ACF were most likely to be bioactive based on the bioactivity score (which is closer to 1), their poor water solubility may challenge the experiment and limit peptide transfer into the bloodstream. A specific solvent may be required during analysis and the peptide solution may influence the DPP-IV inhibitory activity making it inconvenient to work with. Generally, the nontoxic and nonallergenic properties predicted from CVPR were important criteria that were considered.

In vitro characterization and stability of DPP-IV inhibitory peptide

The molecule with the sequence CVPR was synthesized in an attempt to evaluate its DPP-IV inhibitory activity and its stability against pH, temperature, and simulated gastrointestinal digestion (SGID). First, the inhibitory activity of the DPP-IV inhibitory peptide was measured at different peptide concentrations (Figure 4a). Based on the result, the percentage of inhibition increased significantly with higher peptide concentrations, demonstrating the influence of peptide concentration on the inhibitory activity. The IC_{50} of DPP-IV inhibitory peptide was calculated as $1393.61 \pm 28.72 \mu\text{M}$, similar to Atlantic salmon

Table 5. The potential novel peptides to be released from *P. vietnamensis* proteins.

Peptides	CFA (Cys-Phe-Ala)	ACF (Ala-Cys-Phe)	RFPS (Arg-Phe-Pro-Ser)	DEWG (Asp-Glu-Trp-Gly)	NYCL (Asn-Tyr-Cys-Leu)	CVPR (Cys-Val-Pro-Arg)	DACF (Asp-Ala-Cys-Phe)
Potential biological activity	ACE/DPP-IV inhibitor	ACE inhibitor	ACE/ DPP-IV inhibitor	ACE/DPP-IV inhibitor	ACE/DPP-IV inhibitor	ACE/ DPP-IV inhibitor	ACE inhibitor
Bioactivity score	0.941	0.975	0.807	0.516	0.736	0.544	0.923
Enzymes	Pancreatic elastase, leukocyte elastase	Papain, calpain 2	Pancreatic elastase	Pancreatic elastase, stem bromelain	Leukocyte elastase, stem bromelain	Papain, calpain 2	Ficin
MW (g/mol)	339.41	339.41	505.57	505.48	511.59	473.59	454.5
Water solubility	Poor	Poor	Good	Good	Poor	Good	Good
Isoelectric point	2.9	2.97	10.55	0.62	2.86	9.21	0.76
Net charge at pH 7	-0.1	-0.1	1	-2	-0.1	0.9	-1.1
*Resistance to digestion	No	No	No	No	No	No	No
Toxicity	Nontoxic	Nontoxic	Nontoxic	Nontoxic	Nontoxic	Nontoxic	Nontoxic
Allergenicity probability	Nonallergenic	Nonallergenic	Allergen	Nonallergenic	Allergic	Nonallergenic	Nonallergenic

*Resistance to digestion using enzymes: chymotrypsin-low specificity, chymotrypsin-high specificity, pepsin (pH 1.3), pepsin (pH >2), and trypsin.

(*Salmo salar*) gelatin hydrolysate with IC_{50} of $1547.15 \pm 34.15 \mu M$ as reported by Neves *et al.* (2017). According to Liu *et al.* (2019), it is not the length of the peptide that influences the DPP-IV inhibitory activity, but the composition and sequence of amino acid residues might correlate with DPP-IV inhibitory properties. Furthermore, the effect of pH ranges from 3.0 to 11.0 on the relative DPP-IV inhibitory activity of the peptide is depicted in Figure 4b. The inhibitory activity was observed to reduce approaching strong alkaline condition (pH > 9.0), indicating that the peptide favors an acidic condition with the highest relative DPP-IV inhibitory activity at pH 3.0 ($80.10\% \pm 4.64$) and 5.0 ($86.04\% \pm 3.96$). The impacts of different temperatures (30, 50, 70, 90, and $100^{\circ}C$) on the relative DPP-IV inhibitory activity are illustrated in Figure 4c. The highest relative DPP-IV inhibitory activity was observed at $30^{\circ}C$ ($82.19\% \pm 3.30$). Upon increasing temperature, the inhibitory activity of the peptide was gradually decreased. Lastly, the peptide was subjected to two stages of simulated digestion to determine its stability against the activity of gastrointestinal enzymes. Based on Figure 4d, the relative DPP-IV inhibitory activity of the peptide was maintained before or after *in vitro* gastrointestinal digestion.

Discussion

In this study, the proteomic analysis revealed *P. vietnamensis* contained mostly phycobiliproteins. This correlates to a previous study that reported the most abundant protein in red seaweed is phycobiliproteins which consist of phycoerythrin, phycocyanin, and allophycocyanin (Cotas *et al.*, 2020). In the BIOPEP-UWM database, “A” value(s) indicates the frequency of specific proteins containing encrypted bioactive peptides (Minkiewicz *et al.*, 2019). Based on the results, DPP-IV inhibitory activity was predicted as the most potential bioactivity to be released from *P. vietnamensis* proteins followed by ACE inhibitory activity. Similar values of ΣA (1.129–1.482), A_{DPP-IV} inhibitor (0.586–0.688), and A_{ACE} inhibitor (0.336–0.518) were reported from *Gracilaria changii* (Sharmin *et al.*, 2022). These results are supported by *in vitro* findings of Malaysian seaweeds that possessed potent DPP-IV inhibitory activity (Chin *et al.*, 2014). In contrast, green seaweed *Caulerpa spp.* was predicted with high anti-hypertensive properties with a frequency value of ACE inhibitor in the range of 0.3822–0.4330 than DPP-IV inhibitor with a frequency value of 0.0550–0.0714 (Agirbasli and Cavas, 2017).

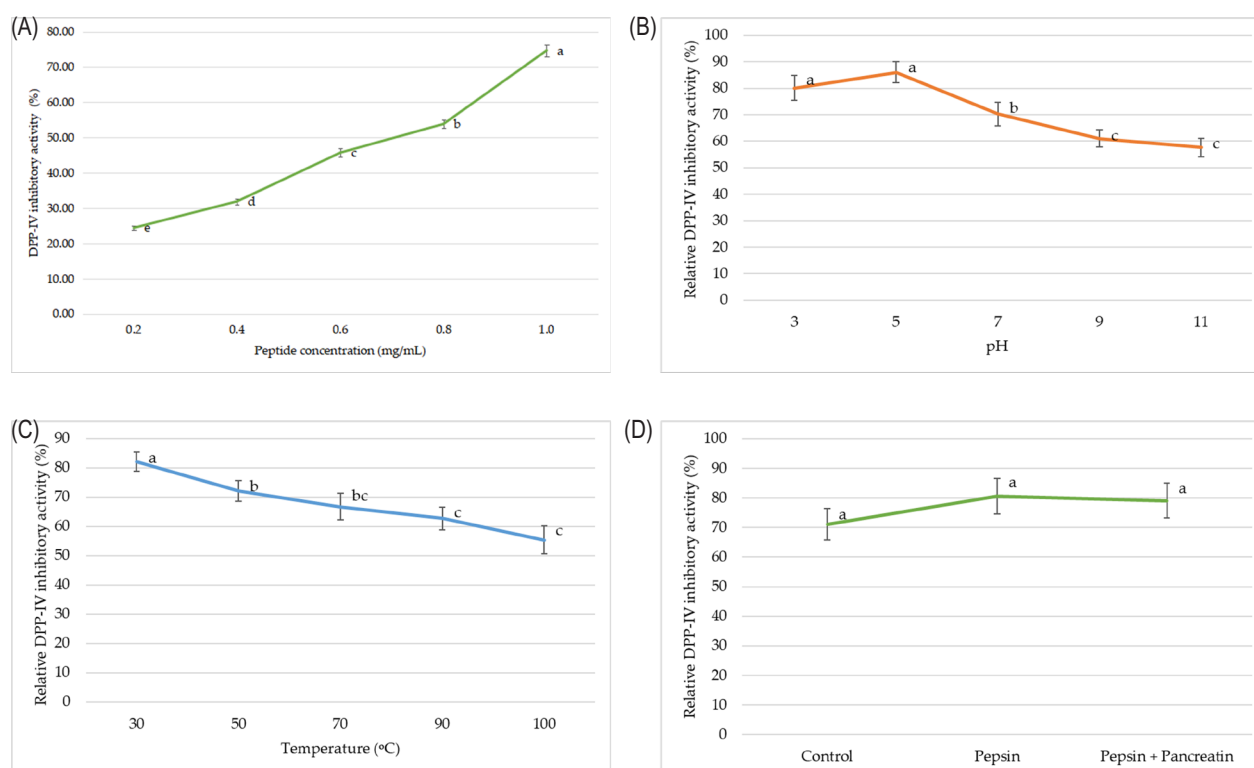


Figure 4. (A) DPP-IV inhibitory activity of peptide at different concentrations; (B) pH stability at pH 3.0, 5.0, 7.0, 9.0, 11.0; (C) Thermal stability at different temperatures (30, 50, 70, 90, $100^{\circ}C$) after 30 min of incubation period; (D) Simulated gastrointestinal stability of peptide against pepsin and pepsin + pancreatin. Data shown are means $\pm$ standard deviation of triplicates, different letters indicate significant differences at $P < 0.05$.

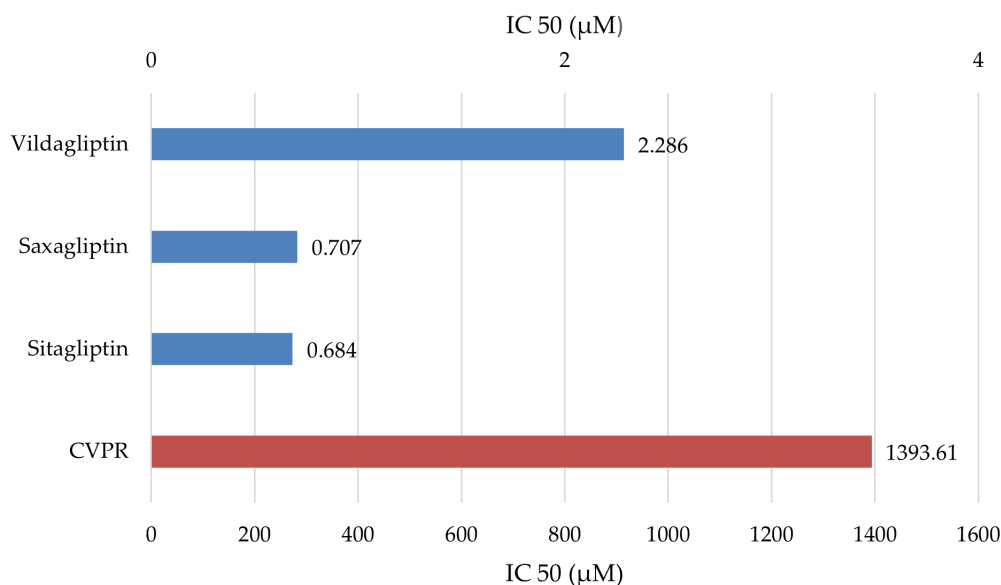


Figure 5. The comparison of IC₅₀ values between peptide and the established DPP-IV inhibitors.

DPP-IV is a serine protease that cleaves two amino acids at the N-terminal, proline, and alanine that possess vital functions in the insulin secretion mechanism. The incretin hormones, glucose-dependent insulino-tropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), are accountable for the role of maintaining the blood glucose level (BGL) by increasing insulin secretion, β -cell mass, decreasing glucagon secretion, and shifting the gastric emptying rate. However, they had a short half-life of less than 2 min and were quickly metabolized by DPP-IV enzyme. DPP-IV inhibition is a primary target in the therapy of type 2 diabetes mellitus (T2DM) which is defined as a failure of the body to govern blood glucose levels efficiently, resulting in impaired insulin secretion as well as insulin resistance. Numerous food proteins have been reported to release DPP-IV inhibitory peptides including from seaweed species such *P. palmata*, *Porphyra dioica*, *Ulva spp.*, and *Gracilaria opuntia* (Cermeño *et al.*, 2019; Cian *et al.*, 2022; Harnedy *et al.*, 2015; Makkar and Chakraborty, 2016). On the other hand, ACE is important in blood pressure management in the renin–angiotensin (RAS) and the kallikrein–kinin systems. The main role of ACE is to convert inactive angiotensin-I to active vasoconstrictor angiotensin-II in the RAS, which results in a rise in blood pressure due to constrictions of blood vessels. The secondary role of ACE is to inactivate the vasodilator bradykinin which is responsible for promoting vasodilation and lower blood pressure. This dual function allows ACE to regulate blood pressure by promoting vasoconstriction (via angiotensin II) and reducing vasodilation (via bradykinin degradation). In people with hypertension, the presence of ACE inhibitors is crucial to lowering angiotensin II levels and increasing

bradykinin, resulting in vasodilation and reduced blood pressure. To date, seaweed species *Acrochaetium sp.* and *Gracilariopsis lemaneiformis* have been reported to possess antihypertensive activity (Deng *et al.*, 2018; Windarto *et al.*, 2022).

In silico proteolysis was utilized as an initial mining of bioactive peptide production as well as to overcome the challenges of *in vitro* and *in vivo* enzymolysis which are time-consuming, costly, and laborious processes (Agyei *et al.*, 2018). In this study, single enzyme treatment was predicted to give a higher score of ΣA_E compared to proteolysis with a combination of enzymes. The lower ΣA_E values obtained may be due to the action of enzyme combinations that would damage the original inhibitory peptides as mentioned by Klompong *et al.* (2007), while less or no bioactive peptides may be obtained from extensive proteolysis. The results suggested calpain 2, papain, pepsin (pH > 2), and stem bromelain as potential enzymes to release peptides with DPP-IV and ACE inhibitory activity. The results were consistent with the published study whereby calpain 2 had maximum A_E for DPP-IV inhibitor from *H. stipulacea* (Kandemir-Cavas *et al.*, 2019). Moreover, papain has been identified as an efficient protease for generating ACE inhibitory peptides from *Ulva lactuca* protein extract (Garcia-Vaquero *et al.*, 2019) and exhibited a great likelihood to release renin inhibitory peptides *in silico* (Pihlanto and Mäkinen, 2017). Meanwhile, pepsin was also reported to release peptides with high antihypertensive activity (Baba *et al.*, 2021). *In silico* proteolysis using pepsin (pH > 2) produced the highest number of bioactive fragments including ACE inhibitor and DPP-IV inhibitor from *Ulva lactuca*. In addition, stem bromelain has been reported to produce

a significant amount of peptide fragments (>100), thus the potential to release bioactive peptides from seaweed (Garcia-Vaquero *et al.*, 2019; Sharmin *et al.*, 2022). Eventually, this *in silico* work helps the researcher to screen for potential enzymes to produce peptides with desired bioactivity.

In the peptide chain, certain amino acids play an important role in inhibiting enzymes such as DPP-IV and ACE due to their specific chemical properties and interactions with enzyme active sites. These amino acids influence the peptide's binding affinity, stability, and inhibitory activity. Specifically, the presence of proline residues at specific positions will make the peptide resistant to DPP-IV's and ACE's cleavage. In contrast, the presence of alanine residues contributes to the peptide's affinity with the DPP-IV active site. Moreover, hydrophobic amino acids, for example, valine and tryptophan enhance the hydrophobic interactions with DPP-IV and ACE, respectively. Basic amino acids such as arginine and lysine are also critical for DPP-IV inhibition where they form strong ionic or hydrogen bonds with DPP-IV. On the other hand, sulfur-containing amino acids including cysteine and methionine enhance and stabilize the peptide–enzyme interaction. These structural–functional relationships are important for the therapeutic and functional applications of bioactive peptides.

Novel tripeptides and tetrapeptides with significant bioactivity potential were reported in this study, and the ones with potential DPP-IV inhibitors were chosen for *in vitro* characterization. Additionally, solubility is one of the crucial aspects to influence the peptides' transfer into the blood, while poor solubility could result in less absorption. Hence, CVPR was chosen to further investigate due to its potency to act as a DPP-IV inhibitor, exhibiting good water solubility, no potential toxicity, and nonallergenic properties. CVPR had proline residues in the peptide chain which may exhibit potent DPP-IV inhibitory activity (Cermeño *et al.*, 2019). In this study, novel peptides were predicted to degrade during digestion. Fundamentally, PepCalc uses simplified models that might not account for the full range of biological factors influencing peptide stability. This model relies on known cleavage motifs and does not fully capture the complex interplay between peptide structure, enzyme specificity, and the environment. Also, it might assume the peptide is fully linear and accessible to enzymes, leading to an overestimation of its susceptibility to cleavage. Hence, it must be aware that this computational tool can sometimes produce false positives for degradation susceptibility due to biases in their training datasets. Nevertheless, this limiting factor could be overcome by an oral delivery system either through the linkage of peptide drugs with lipophilic or hydrophilic moieties, or by encapsulation.

In vitro characterization was carried out to determine the activity and stability of novel peptides associated with the dominant bioactivity predicted *in silico* which is DPP-IV inhibitory activity. To the best of our knowledge, tetrapeptide CVPR has not been previously identified as a DPP-IV inhibitory peptide. CVPR exhibited IC_{50} of $1393.61 \pm 28.72 \mu M$, lower than that of the proline-containing peptide of PP and GGPAGPAV with IC_{50} of 4343.48 ± 29.78 and $8139.11 \pm 134.68 \mu M$, respectively (Neves *et al.*, 2017). This suggests that *P. vietnamensis* has higher DPP-IV inhibitory activity than *Salmo salar*. Nevertheless, *P. palmata* exhibited potent activity of ILAP, LLAP, and MAGVDHI with IC_{50} values of 43.40 ± 1.40 , 53.67 ± 0.82 , $159.37 \pm 13.67 \mu M$, respectively (Harnedy *et al.*, 2015). Currently, gliptins are the most potent commercial DPP-IV inhibitors approved by the Food and Drug Administration (FDA), while there are 10 DPP-IV inhibitor drugs still undergoing clinical trials (Dahlén *et al.*, 2022). According to Gupta *et al.* (2018), the efficiency of gliptins measured as IC_{50} are as of the followings; sitagliptin = $0.684 \mu M$, saxagliptin = $0.707 \mu M$, and vildagliptin = $2.286 \mu M$. Figure 5 compares IC_{50} values between peptide and the established DPP-IV inhibitors. It is noted that lower IC_{50} values indicate potency.

CVPR developed potential inhibitory effects against DPP-IV compared to other peptides with much higher IC_{50} values such as spinach dry-cured ham, milk, and skin or scale collagen-derived peptide with IC_{50} of more than 10,000, 11,000, and 20,000 μM , respectively (Gallego *et al.*, 2014; Hatanaka *et al.*, 2014; Nongonierma and FitzGerald, 2013). It is important to note that *in vitro* IC_{50} does not always predict *in vivo* efficacy accurately. The physiological environment can significantly influence a peptide's activity, and factors such as digestion, absorption, distribution, metabolism, and excretion play crucial roles in determining the actual bioactivity within the body. Generally, peptides with higher IC_{50} values may still contribute to cumulative inhibitory effects when present in complex food matrices, despite their lower individual potency. In the development of nutraceutical products, the activity of CVPR provides a natural and less potent alternative to synthetic drugs, making them suitable for preventive approaches in managing prediabetes or mild hyperglycemia. Interestingly, CVPR may also serve as lead compounds for developing more potent and specific DPP-IV inhibitors and can be used in combination therapies to enhance the efficacy of existing antidiabetic medications.

Nevertheless, analysis of CVPR in animal models and *in vivo* is required to validate its efficiency as a DPP-IV inhibitory peptide for further practical application and commercialization. Previously studied, various dietary proteins found that certain plant-based proteins and

hemoglobin exhibited higher DPP-IV inhibitory activity *in vivo* compared to *in vitro* results. For example, the plasma DPP-IV activity in rats was decreased significantly in the presence of peas, gluten, hemoglobin, and gelatin compared to DPP-IV inhibitory activity *in vitro* (Fleury *et al.*, 2022). Moreover, walnut and tea-derived peptides were observed to improve glucose metabolism in mice *in vivo* (Lu *et al.*, 2019; Mu *et al.*, 2024). This suggests that peptides derived from these proteins may have enhanced efficacy in living systems, potentially due to factors such as improved absorption or stability.

The stability of the peptide against pH, temperature, and SGID was examined. The results of pH stability were similar to the previous finding by Xiang *et al.* (2021) where peptides derived from discarded shrimp heads showed a significant decrease in bioactivity under an adverse alkaline condition. This may be because the peptide is further degraded into an inactive fragment under an extremely alkaline condition, or the pH adjustment might alter the charges in the peptide which eventually affects the activity of the peptide. Furthermore, the thermal stability results are comparable to that of walnut-derived peptides which exhibited good DPP-IV inhibitory activity across different temperatures (Kong *et al.*, 2021). Stability testing of bioactive peptides concerning pH and temperature is crucial for their successful application, especially in nutraceuticals. By testing bioactive peptides under varying pH and temperatures, stability ensures that peptides retain their functional properties, for example, the ability to inhibit enzymes such as DPP-IV or ACE under diverse physiological and processing conditions. Moreover, the thermal and pH stability extends the shelf life of the products, since peptides are often exposed to high temperatures during processing for example, pasteurization, and storage, and may encounter varying pH levels in formulations for example, acidic in beverages and basic in certain supplements. Furthermore, nontoxicity and nonallergenicity are critical for the safe and effective use of peptides in functional foods. Functional foods are intended to provide health benefits beyond basic nutrition, and any potential adverse effects, such as toxicity or allergenicity, can undermine their purpose and marketability. Nontoxic peptides ensure the food does not cause harmful effects, such as cytotoxicity, organ damage, or disruption of normal physiological functions, while non-allergenic peptides minimize the risk of allergic reactions, which can range from mild symptoms to severe. Additionally, these properties ensure regulatory compliance and product acceptance due to functional integrity suitable for human consumption.

For the bioactive peptides to exert their physiological effects within the gastrointestinal tract, circulatory system, and specific cells, the bioactive peptides are anticipated to be resistant to hydrolysis by digestive

enzymes. Figure 4 shows that no significant difference was observed in the DPP-IV inhibitory activity after simulated digestion, indicating that the peptide may resist gastrointestinal digestion. This finding aligns with the results of Harnedy *et al.* (2015), whereby ILAP, LLAP, and MAGVDHI from *P. palmata* were observed to survive an *in vitro* simulated digestion. Other food-derived peptides that have been reported to resist gastrointestinal transit include VPLVM and VPYPQ (Pei *et al.*, 2022; Zheng *et al.*, 2019). These findings suggest that most VP-containing peptides may exhibit stability following SGID. Comparing both *in silico* and *in vitro* results, discrepancies may arise between the two approaches. This is because an *in silico* study is a prediction made based on the *in silico* tools, while an *in vitro* study gives a more real finding in *in vitro* conditions. CVPR might be predicted to degrade by the PepCalc app due to the presence of residues like arginine, which are common targets for proteases. However, the structural features of CVPR for example, the rigidity introduced by proline, potential protective effects of cysteine, and overall conformation may render it more resistant to enzymatic digestion *in vitro* than computational models can predict. Also, *in silico* tools often simulate digestion under standardized conditions for maximum degradation efficiency. Meanwhile, the differences in *in vitro* conditions can influence enzyme activity and the interaction of CVPR with other components in the digestion medium may reduce enzyme access to cleavage sites. Hence, CVPR is not affected by SGID and thus maintains its DPP-IV inhibitory activity. This highlights the importance of validating *in silico* predictions with *in vitro* and *in vitro* assays to account for real-world complexities.

Conclusions

This study found that DPP-IV and ACE inhibitory activities were the most promising bioactivities predicted to be released from *P. vietnamensis* proteins, suggesting their potential to inhibit enzymes involved in the pathologies of diabetes and hypertension. To the best of our knowledge, this is the first study on DPP-IV and ACE inhibitory activity from *P. vietnamensis* proteins via an *in silico* approach. This approach has provided valuable insights for the researcher to predict the most potential bioactivity from *P. vietnamensis*. It has narrowed the selection of potential proteolytic enzymes that could release peptides with a high frequency of DPP-IV and ACE inhibitory activity. Further research on mechanistic studies should be carried out to elucidate the binding interactions between peptides and DPP-IV and ACE through molecular docking and simulation. Also, conducting *in vivo* validation through animal and human studies to assess the real-world efficacy, safety, and pharmacokinetics of these peptides would be useful in designing future

work. *P. vietnamensis* is an attractive renewable resource making it potential for commercial production. It is abundant and sustainable where they are widely available and can be cultivated sustainably in marine environments, with minimal reliance on freshwater or arable land. Furthermore, *P. vietnamensis* offers a wide range of bioactive compounds potential for functional foods, nutraceuticals, and pharmaceuticals. Currently, there are growing consumer preference for natural and sustainable bioactive compounds, thus boosting the commercial viability of seaweed peptides. Nevertheless, the production of bioactive peptides of *P. vietnamensis* at a commercial scale while maintaining their activity and cost-effectiveness is a significant challenge. Hence, the strategies for scalability need to be considered including the optimization of cultivation by the use of biotechnological advances, such as selective breeding and genetic engineering, to improve seaweed yields and peptide content. Moreover, the adoption of advanced scalable extraction techniques to improve yield and reduce costs, as well as the exploration of bioreactor-based methods for controlled peptide production is one of the crucial aspects to be taken into account. Finally, the collaborative efforts between academic institutions, industry, and governments to fund research and accelerate the development of scalable methods would benefit both industries and consumers worldwide.

Authors' Contributions

All the data is available with the authors and shall be provided upon request.

Data Availability Statement

All the data is available with the authors and shall be provided upon request.

Conflicts of Interest

The authors declare no conflicts of interest relevant to this article's content. The funders had no role in the design of the study, collection, analyses or interpretation of data, writing of the manuscript or in the decision to publish the results.

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Supplementary



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CERTIFICATE OF ANALYSIS

Product Name	CVPR
Order ID	U849JIB280_1
Lot No.	U849JIB280-1/PE1488
Sequence	CVPR
Modification	N/A
Length	4AA
Storage	-20°C
Recommended Solvent*	Ultrapure water
Comments	TFA salt

Test Items	Specifications	Results
Molecular Weight	Theoretical MW: 473.59	Consistent
HPLC purity	≥98.0%	99.1%
Appearance	White lyophilized powder	Conforms
Gross Weight	80-100 mg	100.3mg

*Note: Above recommended solvents for reference only. If there is any request for detailed dissolution conditions, we suggest you choose our 'Peptide Solubility Test Service'.

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Certified by: *Li Hui Wei* Date: 03/19/2023

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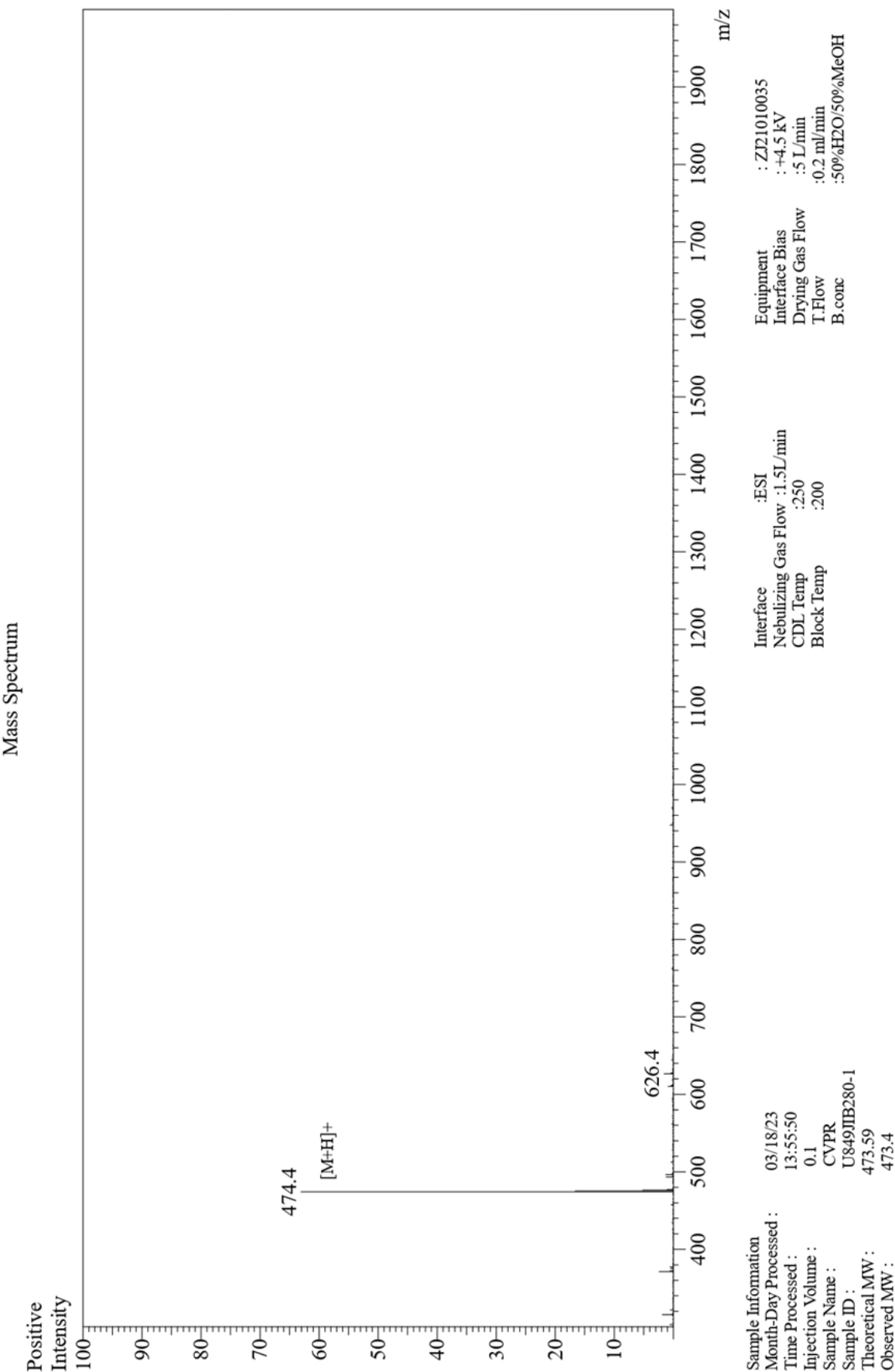
Peptide Qualitative Solubility Test Report

Name	CVPR
Order ID	U849JIB280-1

Solvent	Results
ultrapure water	Soluble
1X DPBS * (pH 7.1±0.1)	Soluble
DMSO	Soluble

DPBS Dulbecco's Phosphate Buffered Saline, containing Potassium Chloride(KCl),Potassium Phosphate monobasic (KH_2PO_4),Sodium Chloride (NaCl)and Sodium Phosphate dibasic ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$);

Comments:
1. The solubility of peptides is largely determined by the polarity of the peptides. Acidic proteins are dissolved in alkaline solutions, basic proteins can be dissolved in acidic solutions, and hydrophobic and neutral polypeptides containing a large number of uncharged polar amino acid residues or hydrophobic amino acids can be dissolved in a small amount of organic solvents first. Then dilute with water. Peptides with higher hydrophobicity are recommended to be dissolved in pure DMSO.
2. Freely soluble: the solvent is added to the sample, the sample dissolves immediately, and the solution is clear and transparent. Soluble: the solvent is added to the sample, the sample dissolves after shaking or sonication, and the solution is clear and transparent. Insoluble: The solvent is added to the sample, the solution is cloudy or flocculent by shaking or sonication. Note: The dissolved concentration of the sample is about 1mg/ml.
3. Peptides containing Cysteine (C), Methionine (M) or Tryptophan (W) are sensitive to oxidation by DMSO. We advise that peptides dissolved in DMSO be used immediately or stored at -20°C (or preferably -80°C) prior to use. Usually, we recommend that the peptides be used in time after dissolving. If the solution peptides need to be stored, it is recommended to store them in small samples to avoid repeated freezing and thawing.
4. When the peptide is insoluble in the solvent of your choice, please refer to the table above for other suggested solvents.
5. Please note that distinct dissolution behaviors may happen between small amounts and large amounts of gross peptide in the same solvent. Generally, larger amounts of peptide take longer to dissolve.
6. The test results are for reference only, and the user needs to choose a suitable solvent according to the experimental needs.
Tested by: Yanyun Shao 03-18-2023



Sample Name :CVPR
Sample ID :U849JIB280-1
Time Processed :14:42:21
Month-Day-Year Processed :03/18/2023

Pump A : 0.065% trifluoroacetic in 100% water (v/v)
Pump B : 0.05% trifluoroacetic in 100% acetonitrile (v/v)
Total Flow:1 ml/min
Wavelength:220 nm

<<LC Time Program>>

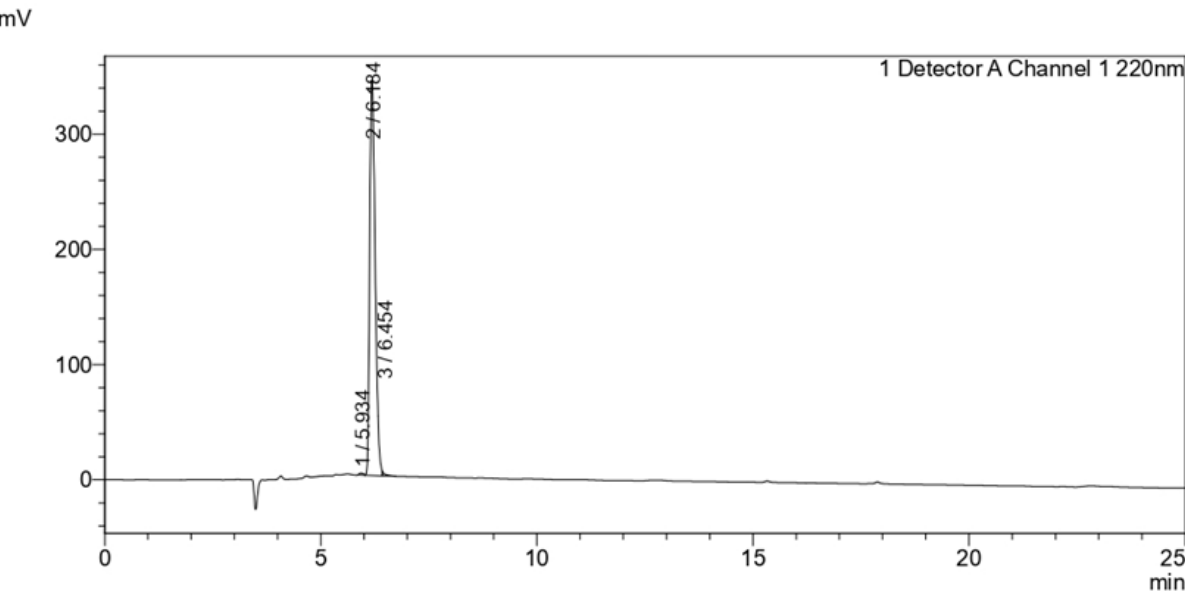
Time	Module	Command	Value
0.01	Pumps	B.Conc	5
25.00	Pumps	B.Conc	65
25.01	Pumps	B.Conc	95
27.00	Pumps	B.Conc	95
27.01	Pumps	B.Conc	5
35.00	Pumps	B.Conc	5
35.01	Controller	Stop	

<<Column Performance>>

<Detector A>

Column :Inertsil ODS-SP 4.6 x 250 mm
Equipment: ZJ19010324

<Chromatogram>



<Peak Table>

Detector A Channel 1 220nm				
Peak#	Ret. Time	Area	Height	Area%
1	5.934	12469	1800	0.408
2	6.184	3030990	337402	99.126
3	6.454	14271	2721	0.467
Total		3057730	341923	100.000